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The genetics of isoflurane-induced developmental neurotoxicity

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ABSTRACT

Introduction: Neurotoxicity induced by early developmental exposure to volatile anesthetics is a characteristic of organisms across a wide range of species, extending from the nematode *C. elegans* to mammals. Prevention of anesthetic-induced neurotoxicity (AIN) will rely upon an understanding of its underlying mechanisms. However, no forward genetic screens have been undertaken to identify the critical pathways affected in AIN. By characterizing such pathways, we may identify mechanisms to eliminate isoflurane induced AIN in mammals.
Methods: Chemotaxis in adult *C. elegans* after larval exposure to isoflurane was used to measure AIN. We initially compared changes in chemotaxis indices between classical mutants known to affect nervous system development adding mutants in response to data. Activation of specific genes was visualized using fluorescent markers. Animals were then treated with rapamycin or preconditioned with isoflurane to test effects on AIN.
Results: Forty-four mutations, as well as pharmacologic manipulations, identified two pathways, highly conserved from invertebrates to humans, that regulate AIN in *C. elegans.* Activation of one stress-protective pathway (DAF-2 dependent) eliminates AIN, while activation of a second stress-responsive pathway (endoplasmic reticulum (ER) associated stress) causes AIN. Pharmacologic inhibition of the mechanistic Target of Rapamycin (mTOR) blocks ER-stress and AIN. Preconditioning with isoflurane prior to larval exposure also inhibited AIN.
Discussion: Our data are best explained by a model in which isoflurane acutely inhibits mitochondrial function causing activation of responses that ultimately lead to ER-stress. The neurotoxic effect of isoflurane can be completely prevented by manipulations at multiple points in the pathways that control this response. Endogenous signaling pathways can be recruited to protect organisms from the neurotoxic effects of isoflurane.
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1. Introduction

Jevtovic-Todorovic et al. and Young et al. demonstrated that commonly used anesthetics caused widespread apoptosis and neuronal degeneration in developing rat brains (Jevtovic-Todorovic et al., 2003; Young et al., 2005). These pathological changes were accompanied by a learning defect that persisted into adulthood in the rat. It is now established that, from nematodes (Gentry et al., 2013) to rodents (Jevtovic-Todorovic et al., 2003; Lu et al., 2006; Yon et al., 2005) and to primates (Creeley et al., 2013; Olsen & Brambrink, 2013), volatile anesthetics *in isolation* are capable of inducing neurodegeneration in the developing nervous system (Jevtovic-Todorovic, 2005; Rappaport et al., 2015; Martin et al., 2014; Creeley et al., 2014). It remains unclear how great a risk anesthetic exposure poses to the newborn human at clinical doses and lengths of time (Rappaport et al., 2015; DiMaggio et al., 2009; Hansen, 2015). However, more than a million children undergo general anesthesia each year in the U.S. (Rabbitts et al., 2010); even rare developmental defects from their use during a critical window of vulnerability have potentially large implications for our current care of children. Since it is impossible to eliminate exposure of neonates to general anesthesia, it is critical that we develop a mechanistic understanding of the process in order to prevent anesthetic-induced neurotoxicity (AIN).

A potential productive approach to understand the mechanism of AIN is a genetic screen to detect the underlying molecular pathways that control its occurrence. In this manner, novel and otherwise unrecognized causes of AIN can be discovered and approaches to prevent AIN may be identified. Using changes in chemotaxis, we previously showed that early exposure to the volatile anesthetic isoflurane is neurotoxic to *C. elegans* (Gentry et al., 2013). Utilizing a genetic approach, we have identified two pathways, highly conserved from invertebrates to humans, that regulate AIN in the nematode, *C. elegans.* Identification of these novel interacting pathways allowed us to completely prevent AIN by genetic and pharmacologic interventions.

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2. Materials and methods

2.1. Strains

N2 Bristol, VC3201 *atfs-1(gk3094)* V, MT1522 *ced-3(n717)*, MT2547 *ced-4(n1162)*, MT5013 *ced-10(n3246)*, MT1082 *egl-1(n487)*, CB 845 *unc-30(e191)*IV, CB1338 *mec-3(e1338)*IV, CB1370 *daf-2(e1370)*III, CB3256 *mab-5(e1751)*III, CF1038 *daf-16(mu86)*I, CW532 *gas-1(fc21)*X, CX4 *odr-7(ky4)*X, CW859 daf-16(mu86);daf-2(e1370), CW860 daf-16(mu86);glr-1(n2461), GA187 *sod-1(tm776)*II, GA416 *sod-4(gk101)*III, GA503 *sod-5(tm1146)*II, GE24 *pha-1(e2123)*III, GS2477 *arIs37* I;*cup-5(ar465)* III; *dpy-20(e1282)* IV, IK105 *pkc-1(nj1)*V, KG532 *kin-2(ce179)*X, KP4 *glr-1(n2461)*III, KU25 *pmk-1(km25)*IV, MC364 *ire-1(ok799)*II, MQ130 *clk-1(qm30)*III, MQ1333 *nuo-6(qm200)*I, MQ989 *isp-1(qm150)*IV, MR507 *aak-2(rr48)*X, MT1522 *ced-3(n717)*IV, MT1976 *unc-86(n946)*III, MT2246 *egl-43(n1079)*II, PY1589 *cmk-1(oy21)*IV, RB967 *gcn-2(ok871)*II (provided to the CGC by the *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation), SJ17 *xbp-1(zc12)*III;zcIs4 V, SJ4005 *zcIs4[hsp-4::GFP]*V, SJ4058 *zcIs9[hsp-60::GFP]*V, SJ4100 *zcIs13[hsp-6::GFP]*V, TJ356 *zIs356[daf-16p::daf-16a/b::GFP + rol-6]* IV, TK22 *mev-1(kn1)*III, TU282 *lin-32(u282)*X, VC1099 *hsp-4(gk514)*II, VC1722 *skn-1(ok2315)*IV/*nT1 [qIs51]*(IV;V) (provided to the CGC by the *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation), VC433 *sod-3(gk235)*X, VC498 *sod-2(gk257)*I, CW645 *sod-2(gk257)*;*sod-3(gk235)*, VM487 *nmr-1(ak4)*II, and ZG31 *hif-1(ia4)*V were all obtained from the *Caenorhabditis* Genetics Center, Minneapolis MN. *gas-1(fc21)* was isolated in our laboratory (Kayser et al., 2001). CW859 (*daf-16;gir-1*) and CW860 (*daf-16;daf-2*) were constructed by crossing *daf-16(mu86)* with CB164 *dpy-17(e164)* to make *daf-16;dpy-17* before crossing into *glr-1(n2461)* or *daf-2(1370)* respectively. The resulting strains were allowed to self-fertilize to remove *dpy-17*. All mutations were confirmed by sequencing. All strains were grown as previously described on agar plates containing nematode growth media (NGM) with the *E. coli* strain OP50 as food (Brenner, 1974).

2.2. Synchronization

For each assay, cohorts of worms were synchronized on 35 mm NGM plates by limiting egg laying to 2–4 h at 20 °C and then grown for 20 h at 15 °C or 20 °C as necessary to obtain newly hatched L1 animals. The plates of newly hatched L1 animals were either exposed to isoflurane as described below or held at 15 °C to slow development to match the isoflurane exposed cohorts.

2.3. Isoflurane exposure

C. elegans L1 larvae were exposed to isoflurane at their clinical EC_{95} (~6.5% isoflurane) for 4 h at 20 °C beginning at 20 h after being laid as eggs. Generally, these animals were at hours 4–8 of L1 development. Some mutants developed slowly, such that the time of isoflurane exposure was adjusted to approximate hours 4–8 of normal L1 development. Isoflurane concentration was checked by gas chromatography as previously described (Morgan et al., 1990). Control animals were kept in room air at 15 °C during that hour to slow development to match isoflurane exposure. After exposure, experimental and control animals were cultured at 20 °C and tested for chemotaxis 3–5 days later, depending on the mutant strain, on day one of adulthood.

2.4. Chemotaxis

Young adult worms were washed three times in chemotaxis buffer (5 mM potassium phosphate, 1 mM calcium chloride, and 1 mM magnesium sulfate) before being transferred in a minimal volume in the center of a 9 cm NGM assay plate. The plates were divided into 3 regions in a modification of the technique described by Bargmann (Gentry et al., 2013; Bargmann & Horvitz, 1991). One region contained an attractant (a 20ul spot of OP50) while the opposite region contained no attractant. The middle region served as the starting point for the animals. 2–3 plates of both control (unexposed) and exposed animals were assayed in parallel on a given day. Worms in each region were counted 1 h after transfer. Scoring was done by an observer blinded to the exposure state of the strain but not to the strain being studied. A chemotaxis index (CI) was calculated using the formula: CI = 100× (worms at food side – worms at control side) / total. All results reported are new for this study (earlier results for N2, *ced-3* and *gas-1* were not included in this study).

2.5. Isoflurane preconditioning

Within 1 h after hatching, synchronous L1 s were exposed to 6.5% isoflurane for 1 h, then allowed to recover for 3 h, before being exposed to isoflurane as per the usual protocol. Control animals were kept in room air at 15 °C during that hour (to slow development to match isoflurane exposure). They were tested as adults as described above in the chemotaxis experiments. All preconditioning assays were performed in duplicate for control and exposed animals.

2.6. Rapamycin

Rapamycin (LC laboratories) was dissolved in 100% dimethyl sulfoxide (DMSO) at 50 mg/ml and added to plate agar to 100 uM with final DMSO concentration of 0.2% as described by Robida-Stubbs et al. (Robida-Stubbs et al., 2012). Control plates contained 0.2% DMSO. Egg laying hermaphrodites were placed on rapamycin or DMSO plates, both with OP50, for 2 h for synchronization, and then removed, as described above in the Synchronization section. Eggs were kept on rapamycin or DMSO plates until hatching and then exposed to isoflurane as L1 s as described above. L1 animals were transferred to OP50 plates without rapamycin or DMSO the morning following isoflurane exposure, approximately 24 h after hatching and 16 h after isoflurane exposure.

2.7. Statistical analysis

Chemotaxis indices (CIs) are the mean of 6–9 experiments (except for N2 where $n = 15$) each containing >50 animals (total animals for each strain, 300–450, except N2, total > 800). Errors for CI are reported as the standard errors of the mean (SEM). Changes in CI (ΔCI) between exposed and unexposed animals of a given genotype are calculated as the mean difference between the CI of the unexposed and the exposed animals. Error bars for ΔCI were calculated by combining the standard deviations (SD) of the CIs and then calculating the SEM from the SD. Values for ΔCI were compared by one-way ANOVA. If a significant difference was identified by ANOVA, then each mutant strain was compared to N2. Significance was defined as $p < 0.01$.

Fig. 1. A, C, E, G, I. Chemotaxis indices (CIs) in adults after exposure to isoflurane as L1 larvae. Unexposed animals (solid fill), exposed animals (angled hatching). For all graphs, error bars denote SEM values, $N > 300$ animals for each value. B, D, F, H, J. Differences in CIs (ΔCI) between exposed and unexposed animals. Difference between ΔCI of N2 (19.5 ± 3.7) and each mutant was compared to determine if the mutant affected AIN. ** = ΔCI different from N2, $p < 0.01$, *** = ΔCI different from N2, $p < 0.005$. A and B. Mitochondrial mutants. Chemotaxis in unexposed mitochondrial mutants (*gas-1*, *nuo-6*, *mev-1*, *isp-1*) was not worsened by isoflurane exposure. The exception was *clk-1* which had a ΔCI similar to that of N2. C,D. ROS scavengers/ER Stress. The effects of defects in ROS scavenging on AIN in *C. elegans*. CIs of five superoxide dismutase mutants and *hsp-4* (loss of the ER-specific heat shock protein HSP-4). E and F. DAF-2 dependent pathway. The effects of the *daf-2* stress pathway on AIN. Loss of DAF-2 removed the AIN effect. The *daf-16* mutation removed the effect of *daf-2* on AIN. G and H. Kinases. Effects of 5 kinases on neurotoxicity. Loss of *cmk-1* and *gcn-2*, both involved in innate immunity and ER-related stress, eliminated AIN. Loss of *ire*-1 also eliminated AIN and is discussed later. I and J. Transcription factors. The transcription factors *skn-1*, *hif-1* and *xpb-1* all eliminated AIN.

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